Effects of Dimethyl Sulfoxide and Polycationic Neomycin on Stimulation of Purified Plasma Membrane Ca^{2+}-Pump by Negatively Charged Phospholipids

J. LEHOTSKÝ[1], L. RAEYMAEKERS[2] and R.CASTEELS[2]

1 *Comenius University, Jessenius Medical School, Department of Biochemistry, 036 01 Martin, M. Hora 4, Czechoslovakia*
2 *Laboratory of Physiology, K. U. Leuven B-3000 Leuven, Belgium*

Abstract. At least two reaction steps are involved in the activation of purified plasma membrane Ca^{2+}-transport ATPase by negatively charged phospholipids depending on the type of phospholipids (Lehotský et al. 1992). The effect of negatively charged phospholipids on Ca^{2+}-stimulated ATPase (cycling activity) was compared with that of *p*-nitrophenylphosphatase (E_2-form activity) catalyzed by Ca^{2+}-pump. PIP like PS, activated Ca^{2+}-ATPase activity by modifying ATP activation curve with increasing V_{max} of the high affinity site. Ca^{2+} -ATPase activity reconstituted in PC was stimulated by DMSO(10%) by a factor of 1.36. The activity stimulation by DMSO was only weak in PS and activity was inhibited in PIP. Also, phosphatase activity catalyzed by Ca^{2+}-pump was strongly stimulated by DMSO and was differentially affected by phospholipid head group. Positively charged neomycin (5 mmol/l) had no effect on Ca^{2+}-ATPase activity reactivated in PC or PS, but the stimulatory action of PIP was suppressed. Relative stimulation of phosphatase activity by PS was not influenced. Both hydrolytic activities catalyzed by Ca^{2+}-transport ATPase were differentially affected by organic solvents and polycations with respect to the kind of the phospholipid.

Key words: Plasma membrane — Ca^{2+}-transport ATPase — Phospholipid stimulation

Introduction

The calcium pumping ATPase of the erythrocyte plasma membrane is responsible for calcium homeostasis in these cells. This enzyme can be activated by the Ca^{2+}-calmodulin complex, by self-association of ATPase molecules, by partial proteolysis, and by negatively charged phospholipids (for review see Carafoli 1991; Missiaen et al. 1991). However, little is known about how these agents activate the Ca^{2+}-

pump. Data from different laboratories indicate that the binding of calmodulin to the regulatory domain and modulation of the enzyme by acidic phospholipids or by self-association is mediated by hydrophobic interactions (James et al. 1988; Benaim and de Meis 1990; Kosk-Kosicka and Bzdega 1990).

Enyedi et al. (1987) postulated the existence of two regulatory domains, one responsible for calmodulin activation and the other one mediating the effect of negatively charged phospholipids. A correlation was found between the number of negative charges on the phospholipid molecules (PIP 2 > PIP >PI = PA = PS) and their potency in stimulating ATPase activity (Missiaen et al. 1989*b*). The importance of the number of negative charges was established more directly using fluorescence energy transfer as a measure of molecular interaction between phosphoinositides and the ATPase protein (Verbist et al. 1991). From circular dichroism experiments and fluorescence measurements it was concluded that PS induces a change in the secondary structure of ATPase, primarily in its α-helical content (Wrzosek et al. 1989).

In our previous work it was shown that at least two reaction steps are involved in the activation of the purified Ca^{2+}-pump by negatively charged phospholipids. These steps are differentially affected depending on the phospholipid species, resulting either in an acceleration of the phosphoprotein formation in the presence of PIP, or in a faster dephosphorylation in the presence of PS (Lehotský et al. 1992).

The objective of the present study was to compare the effects of negatively charged phospholipids on ATPase and on the *p*-nitrophenylphosphatase activities catalyzed by purified Ca^{2+}-transporting ATPase. The results indicate that these activities are differentially affected by organic solvents and polycations with respect to the kind of the phospholipid.

Abbreviations: (Ca^{2+} + Mg^{2+})-ATPase — (Ca^{2+} + Mg^{2+}) activated ATPase; PI — phosphatidylinositol; PA — phosphatidic acid; PIP — phosphatidylinositol 4-phosphate; PS — phosphatidylserine; PC — phosphatidylcholine; *p*NPP — *p*-nitrophenyl phosphate; DMSO — dimethylsulfoxide; EGTA — ethyleneglycol-bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid.

Materials and Methods

Chemicals

Phosphoenolpyruvate, pyruvate kinase, lactate dehydrogenase, *p*-nitrophenyl phosphate, ATP were obtained from Boehringer (Manheim, FRG). PC, PS, PIP from bovine brain, PIP2 from chicken eggs, NADH, neomycin sulfate, dimethylsulfoxide (DSO) were all from Sigma chemicals (USA); octaethyleneglycol dodecyl ether ($C_{12}E_8$) was from Calbiochem (USA).

Preparation of erythrocyte ghost membranes

Erythrocyte ghosts were prepared from fresh porcine blood according to Steck and Kant (1974)

Purification of (Ca^{2+} +Mg^{2+})-ATPase

The plasma membrane Ca^{2+}-transporting ATPase from erythrocytes was purified using a modification of the calmodulin-affinity chromatography as described by Kosk-Kosicka et al (1986) and Missiaen et al (1989*b*) This procedure allows purification of the enzyme in the absence of phospholipids Briefly, erythrocyte ghosts were centrifuged in a Beckman Ti60 rotor at 50,000 r p m (254, 400 × g_{max}) for 30 min and resuspended in 130 mmol/l KCl, 20 mmol/l Hepes (pH 7 4), 0 5 mmol/l $CaCl_2$, 2 mmol/l dithiothreitol and 20% glycerol at 8 mg membrane protein/ml The membranes were solubilized by adding 4 mg $C_{12}E_8$ per ml of buffer After 10 min of incubation at 4 °C under continuous stirring, the nonsolubilized material was removed by centrifugation in a Ti60 rotor at 50,000 r p m /min for 30 min The $C_{12}E_8$ solubilized material was added to a calmodulin Sepharose 4B affinity gel that had been equilibrated with buffer *A* (130 mmol/l KCl, 20 mmol/l Hepes, pH 7 4, 1 mmol/l $MgCl_2$, 0 1 mmol/l $CaCl_2$, 2 mmol/l dithiothreitol, 20% glycerol and 0 4% $C_{12}E_8$) After incubating this mixture for 1 h at 4 °C, the gel was transferred into a chromatography column The unbound protein was removed by washing the column with 10 volumes of buffer *A* Thereupon the column was washed with 10 volumes of buffer *A* (the same as *A* but containing 0 05% $C_{12}E_8$ rather than 0 4% Finally, (Ca^{2+} + Mg^{2+})-ATPase bound to the column was collected by washing the gel with the same buffer as above except that 0 1 mmol/l $CaCl_2$ was replaced by 2 mmol/l EDTA The (Ca^{2+}+Mg^{2+})-ATPase preparation was finally concentrated in Centriprep 10-Concentrator tubes(Amicon) Different preparations were pooled and stored at −80 °C The final concentration was 200 μg/ml A molecular mass of M_r = 138,000 for the (Ca^{2+}+Mg^{2+})-ATPase as determined by SDS-gel electrophoresis was used to calculate molar amounts of the protein

Reconstitution of the (Ca^{2+} +Mg^{2+})-ATPase

The (Ca^{2+}+Mg^{2+})-ATPase purified as described above did not present any detectable enzyme activity, but the (Ca^{2+}+Mg^{2+})-ATPase could be reactivated with phospholipids in a total lipid to ATPase ratio of 100 mol/mol This ratio was obtained by adding 50 μl of the ATPase preparation to 2 5 μl lipid mixture and Vortex mixing Lipid mixtures were made from stock solutions of phospholipids in chloroform/methanol (7 1, v/v) The solvent was evaporated under a stream of nitrogen, and the lipids were redissolved in the (Ca^{2+}+Mg^{2+})-ATPase buffer (130 mmol/l KCl, 20 mmol/l HEPES (pH 7 4), 1 mmol/l $MgCl_2$, 2 mmol EDTA 0 05% $C_{12}E_8$ and 20% glycerol (v/v) and sonicated for 6 5 s The final lipid concentration was 5 mg/l

Measurement of (Ca^{2+} +Mg^{2+})-ATPase and p-nitrophenylphosphatase activities

The standard assay medium for measurements of the (Ca^{2+}+Mg^{2}+)-ATPase activity had the following composition Imidazole -HCl (pH 6 9), 30 mmol/l, $MgCl_2$, 6 mmol/l, KCl, 100 mmol/l, EGTA, 0 4 mmol/l, NaN_3,5 mmol/l, NaCl, 20 mmol/l, phosphoenolpyruvate, 1 5 mmol/l, pyruvate kinase, 40 U/l, lactate dehydrogenase, 40 U/l, NADH, 0 26 mmol/l The ATP concentration is indicated in the Figure legends Ca^{2+} was added to yield 10 μmol/l ionized Ca^{2+} Free Ca^{2+} concentrations were calculated by a computer program Cabuf, generously provided by Dr G Droogmans (K U Leuven) The assay was started

by transferring 20 μl of ATP (about 4 μg protein) to 980 μl assay medium at 37 °C and measuring the decrease in absorbance at 340 nm.

Phosphatase activity of Ca^{2+}-transporting ATPase was quantified at 37 °C as *p*-nitrophenylphosphatase activity (*p*NNPase) by the release of *p*-nitrophenol which was detected spectrophotometrically at 430 nm. The reaction medium had the following composition: HEPES-KOH (pH 7.4), 30 mmol/l; KCl, 100 mmol/l; NaCl, 10 mmol/l; $MgCl_2$, 6 mmol/l; EGTA, 0.4 mmol/l; NaN_3, 5 mmol/l; *p*-nitrophenylphosphate, 10 mmol/l. In case of neomycin, controls contained equimolar K_2SO_4 instead of neomycin sulphate.

Antibody (MAb 2B3) against plasma membrane Ca^{2+} pump was purified from mouse ascites fluid by anion-exchange chromatography (used as a generous gift of Dr. Jan Verbist, K. U. Leuven).

Protein was determined by the bicinchoninic acid (BCA) Protein Assay Reagent kit obtained from Pierce (Rockford, Ill., USA). Bovine serum albumin was used as a standard.

The V_{max} and K_m values were calculated from the ATP activation curves using the Enzfitter (Version 1.02) computer program (Elsevier Biosoft). The data were fit using equation (1) from Rossi and Rega (1989).

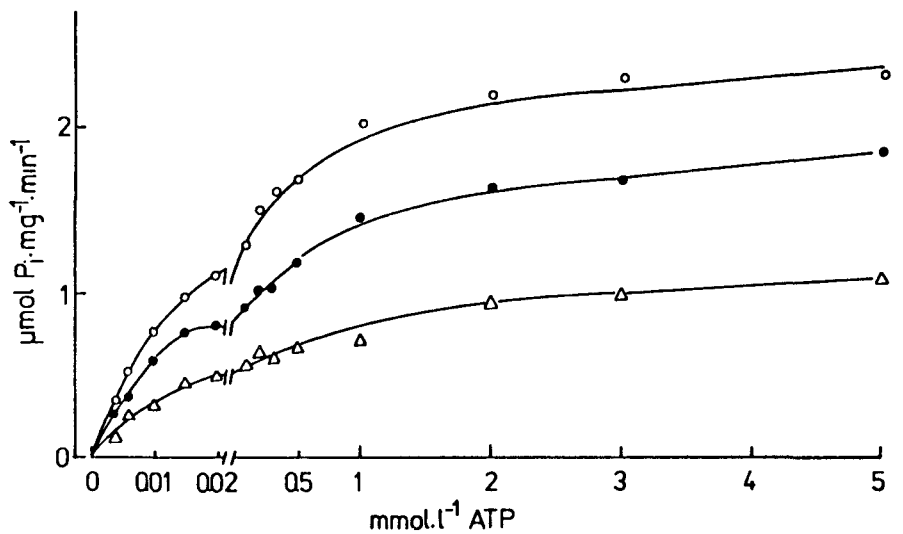

Figure 1. ATP activation curve of Ca^{2+} stimulated ATPase activity of the plasma membrane Ca^{2+}-pump from porcine erythrocytes reactivated by negatively charged phospholipids. The ATPase was reactivated by adding 5 μl of a lipid mixture (5 mg/ml stock) to 100 μl of ATPase (approx. 200 μg/ml). The lipid was either pure PC (Δ) or a mixture of 20% PS (•) or PIP (o) and 80% PC. The total amount of lipid was kept constant. To obtain the indicated concentration, ATP was added in a cumulative way. The free Ca^{2+} and Mg^{2+} concentrations were 10 μmol/l and 1.4 mmol/l, respectively. The experimental points represent the means of 3–5 determinations in 3 different ATPase preparations. The S.E.M. ranges between 5–10% of the means.

Results

Fig. 1 shows the ATP activation curves for purified and lipid reactivated Ca^{2+} transporting ATPase from porcine erythrocytes. Acidic phospholipids PS and PIP (at 20 percent of total added, the remainder being PC) increased the V_{max} of the high affinity component by a factor of 1.6 and 2.25 respectively, as compared to pure PC, without significantly affecting K_m. K_m for the low affinity component was higher than 5 mmol/l ATP in PC and decreased to 1.8 ± 0.17 mmol/l in PS and to 1 ± 0.12 mmol/l in PIP. The difference between PS and PIP in V_{max} calculated for the low affinity component was not significant. The effect of PS on the purified ATPase from porcine erythrocytes shown in Fig. 1 is very similar to that described by Rossi and Rega (1989) on the membrane bound ATPase of human erythrocytes. In addition, our results show a stronger effect of PIP than PS.

Table 1. Effect of purified monoclonal antibody 2B3 on Ca^{2+} stimulated ATPase activity of the erythrocyte Ca^{2+} pump reconstituted in 20% of negatively charged phospholipids.

	Ca^{2+}-ATPase activity (% of inhibition)			
	0.05 mmol/l ATP	0.5 mmol/l ATP	Nonimmune IgG 0.05 mmol/l ATP	Nonimmune IgG 0.5 mmol/l ATP
PC	34 ± 1.6	20.5 ± 2.8	1.2 ± 0.04	0.9 ± 0.06
PS	31 ± 3.2	19.0 ± 2.4	0.6 ± 0.03	1.07 ± 0.09
PIP	28 ± 2.7	22.0 ± 3.6	0.7 ± 0.06	0.8 ± 0.07

The NADH coupled system assay at a free Ca^{2+} concentration of 10 μmol/l was used to quantify inhibition of Ca^{2+}-ATPase activity. A 4 μg sample of Ca^{2+} pump was preincubated in the reaction mixture in a final volume of 1 ml (with 10 μg/ml antibody 2B3 or 10 mg/ml nonimmune IgG) for 1 h at room temperature. The reaction was started by adding ATP. The results are means $\pm$ S.E.M. of 3–5 determinations in 3 different ATPase preparations.

A monoclonal antibody (MAb 2B3) directed against the Ca^{2+} transporting ATPase from the plasma membrane was used. This antibody was shown to react with the putative functionally important site of the Ca^{2+}-pump protein because it acted as a competitive inhibitor of ATP (substrate) binding (Verbist et al. 1986). Tab. 1 shows the effect of MAb 2B3 on the Ca^{2+}-ATPase activity of the Ca^{2+}-pump from porcine erythrocytes reactivated in different acidic phospholipids. In the presence of all phospholipids used, the antibody was found to inhibit ATPase activity to the same extent (approximately 30 percent). As described earlier, the

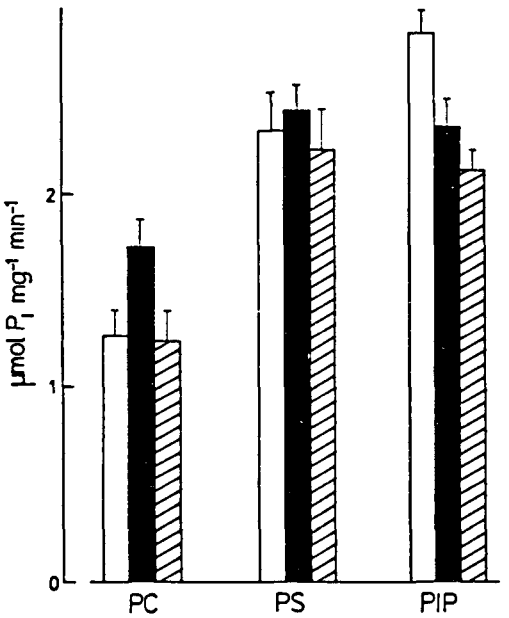

Figure 2. Effect of hydrophobic DMSO (10%) (▮) and polycationic neomycin (5 mmol/l) (▨) on Ca^{2+}-stimulated ATPase activity (control □) of purified erythrocyte Ca^{2+}-pump reconstituted in 20% of negatively charged phospholipids In the presence of DMSO, activity was measured at 10 μmol/l free Ca^{2+} by the release of $^{32}P_i$ from ^{32}P-ATP, by the method described by Benaim and de Meis (1990) Controls were the same except DMSO In case of neomycin, NADH coupled enzyme system assay was used and controls contained 5 mmol/l K_2SO_4 instead of neomycin sulfate The results are expressed as means ± S E M of 5 determinations in 3 different ATPase preparations

inhibiting effect depends on ATP concentration, but no changes in relative inhibition, expressed as the percentage of activity in pure PC, were observed

Fig 2 illustrates the effect of positively charged compound neomycin on Ca^{2+}-ATPase activity of Ca^{2+}-pump prepared from porcine erythrocytes The results are similar to those obtained on smooth muscle (Missiaen et al 1989a) Neomycin had virtually no effect on ATPase activity reactivated by pure PC or by 20 percent PS, however, as expected, the stimulatory action of PIP (as compared to that of PC) was substantially suppressed

Like other P type ATPase, the Ca^{2+} transporting ATPase from plasma membrane catalyses the hydrolysis of *p*NPP (Carafoli 1991) The *p*NPPase activity of the Ca^{2+}-pump reconstituted from porcine erythrocytes was stimulated in a dose dependent manner by PS but was not affected by PIP (Lehotský et al 1992) As can be seen from Fig 3 positively charged neomycin decreased the *p*NPPase

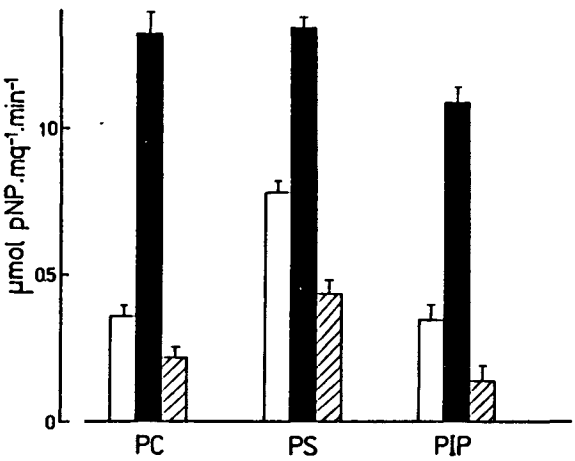

Figure 3. Effects of 10% DMSO (▮) and 5 mmol/l neomycin (▯) on *p*-nitrophenylphosphatase activity (control ▨) of plasma membrane Ca^{2+}-pump reconstituted in 20% of negatively charged phospholipids. The *p*NNPase activity was measured as described in Materials and methods section. Controls were the same except DMSO. In case of neomycin, controls contained 5 mmol/l K_2SO_4 instead of neomycin sulfate. The values represent means ± S.E.M. of 5 different observations.

activity in all reactivated phospholipids by about 40 percent with a more selective inhibition in PIP. However, it did not influence the relative stimulation by PS expressed as percentage of the activity in PC.

It has been postulated by the de Meis group (de Meis 1989) that the phosphate acceptor site of "*P*"-type Ca^{2+}-pumps resides in a hydrophobic pocket. Therefore, during the backward reaction of the ATPase when phosphate is transferred to the enzyme the activity is strongly facilitated by organic solvents which reduce the polarity of the medium. Inclusion of the hydrophobic compound DMSO into the reaction medium increased V_{max} and also the Ca^{2+} affinity of the Ca^{2+}-pump purified from human erythrocytes in PC to values reported for calmodulin (Benaim and de Meis 1989; 1990). However, by testing the influence of DMSO on Ca^{2+}-ATPase activity of the porcine Ca^{2+}-pump reconstituted in the presence of acidic phospholipids we observed remarkable differences in activation effects. In pure PC, 10% DMSO maximally stimulated the activity of Ca^{2+}-ATPase (by a factor of 1.36), whereas there was a weaker stimulation in PS (by a factor of 1.04), and in PIP hydrophobic DMSO had a weaker inhibitory effect (by a factor 0.83). Although it can be supposed that in the presence of DMSO the environment of the catalytic site became more hydrophobic, there still are some differences between the stimulatory effects of the individual phospholipids.

Higher concentrations of DMSO (15–30%) were shown to activate Ca^{2+}-ATPase only in PC. In both acidic lipids this concentration showed inhibition of the stimulatory effect on the ATPase activity (results not shown). Moreover, *p*NPPase activity which represents the E2 form of the Ca^{2+}-pump protein, is strongly stimulated by 10 or 20 percent DMSO in reaction medium. In this condition the *p*NPP-ase activity is no longer stimulated by PS as compared to PC. There still persists a differential effect of PS versus PIP, and the activity in the presence of PS remains higher than in that of PIP. Probably due to its more positive charges PIP becomes inhibitory if compared to PC.

Discussion

Activation of the plasma membrane Ca^{2+}-transport ATPase by negatively charged phospholipids has been found to increase both V_{max} and the affinity for Ca^{2+} (Missiaen et al. 1989*b*). Also, it has been shown that the number of negative charges of phospholipids is important for both the binding and the potency to stimulate activity of the Ca^{2+}-pump (Missiaen et al. 1989*b*, Verbist et al. 1991). Little is known, however, about the site/s of interaction with the enzyme or about the elementary reaction steps of the ATPase cycle which are influenced by these lipids.

In our previous work based on phosphointermediate levels and *p*NPPase activity measurements, we have been the first to prove that at least two reaction steps are involved in the activation depending on the type of phospholipids. PIP affected a step which leads to the accelerated formation of the phosphointermediate whereas PS stimulates two reaction steps: one up-stream and one down-stream of the intermediate. Another possible mechanism was suggested by Rossi and Rega (1989) who have shown for the membrane-bound ATPase that PS modifies the ATP activation curve comprising two components. PS increased V_{max} of the high affinity site. In the present study we obtained similar results with purified, delipidated and reactivated ATPase. Moreover, we could find a similar effect of PIP on the ATP activation curve with PIP having a stronger effect than PS. It seemed from these results that PS and PIP exert their action on ATP dependence of ATPase by a common mechanism.

A monoclonal antibody 2B3 was raised to test whether the inhibitory effect of the antibody is affected by acidic phospholipids. The 2B3 antibody was found to inhibit ATPase activity to the same extent in all phospholipids. Although the antibody seemed to react as a competitive inhibitor of ATP binding in the putative functionally important site (Verbist et al. 1986), this site is probably not directly influenced by added phospholipids.

The reaction steps whereby the plasma membrane Ca^{2+}-transport ATPase catalyzes *p*NPP hydrolysis are not fully understood. This hydrolysis is strongly

influenced by the experimental conditions such as Ca^{2+} or ATP concentration (Verma and Peniston 1984; Rossi et al. 1986; Rossi and Caride 1991). It involves only a part of the ATPase reaction cycle and probably occurs at the low affinity binding site which is exposed when the enzyme is in E2 conformation (Rossi et al. 1986). Although possibly, it includes additional reaction steps which are not shared by the ATPase cycle, the finding that the acidic lipids, which do not increase the phosphointermediate level (PS, PI, PA), increase *p*-NPPase activity whereas PIP and PIP_2 which increase E-P level do not, might be considered as indicative of a differentiation between phospholipids action on the reaction cycle (Lehotský et al. 1992). We now report that positively charged neomycin did not affect the stimulation of *p*NPPase activity by PS. Also, we showed that there was a rather high selectivity of neomycin to inhibit the action of PIP on Ca^{2+}-ATPase, but the selectivity was not as strong as that to less negative PS. These results are similar to those of Missiaen et al. (1989*a*) obtained with smooth muscle plasma membrane Ca^{2+}-pump. The selectivity of neomycin in inhibiting the action of PIP_2 and PIP but not of PS could be explained in at least two ways. One possibility is that electrostatic interactions between neomycin and lipids possessing only one charge (PS) are to weak. Alternatively, the action of PS could primarily be determined by its hydrophobic moiety. However, the latter possibility is not favored as the effect of PI from bovine brain is similar to those of PS and PIP (bovine brain as well), and no difference was seen between the effects of PS isolated from bovine brain and chicken eggs (results not shown).

The reduction of water activity at the active site of the Ca^{2+}-ATPase by organic solvents, which leads to the decrease of the free energy of hydrolysis of phosphointermediate, strongly activated the Ca^{2+}-ATPase activity of the human red blood cell Ca^{2+}-pump purified in pure PC (Benaim and de Meis 1989; 1990). Inclusion of DMSO into the reaction medium in their experiments increased V_{max} as well as Ca^{2+} affinity in a manner similar to that observed with calmodulin. This was explained by postulating a role of hydrophobic interactions in the modulation of the Ca^{2+}-pump both for calmodulin and phospholipids (Benaim and de Meis 1990). Thus, organic solvents can be expected to mimic the effects of both agents; however, this is accomplished by interactions with different domain(s) of the protein.

By testing the influence of DMSO on our purified delipidated and reconstituted porcine Ca^{2+}-pump in acidic phospholipids we observed differences in the effects on both Ca^{2+}-ATPase and *p*NPP-ase activities. Although the differences in further stimulation of Ca^{2+}-ATPase activity in DMSO by phospholipids are not so great as in control experiments (without DMSO) we could not observe in pure PC stimulation to the same extent as that of PS or PIP. Even activation by PIP in DMSO was significantly decreased as compared to the stimulatory effect of PIP itself. We assume that the hydrophobic environment is very important for the stimulation of Ca^{2+}-ATPase activity by acidic phospholipids. Additionally, the

phospholipid head group must play some role as well. The nature of such an interaction could be controlled by negative charges of phospholipids or by factors other than simple electrostatic, e.g. steric interaction and hydration (Szymanska et al. 1991). An argument in favor of this is the observation that individual head groups also affect strong DMSO stimulation of *p*NPPase activity in a different manner. No stimulation was shown in PS as compared to PC. In PIP the activity became even more inhibitory than that of PC.

In summary, the present study extends our previous observations (Lehotský et al. 1992) and indicates that acidic phospholipids differentially affect Ca^{2+}-ATPase (cycling activity) and *p*-nitrophenylphosphatase (E_2 form activity) also in the presence of both positively charged neomycin and hydrophobic organic solvents. Likely, this can be explained by the fact that acidic phospholipids modify more than one reaction step of the ATPase cycle. Lysine-rich region has been proposed as a binding site for acidic lipids (Zvaritch et al. 1990), and also arginine residues were shown to play a role in the stimulatory effect (Missiaen et al. 1989c) but the exact nature of the interaction between protein and phospholipids is still far from being understood.

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